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in FGF/MAPKK-Mediated Mesoderm Induction in *Xenopus*

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Recent studies on *Xenopus* development have revealed an increasingly complex array of inductive, prepatterning, and competence signals that are necessary for proper mesoderm formation. In this study, we establish that fibroblast growth factor (FGF) signals through mitogen-activated protein kinase kinase (MAPKK) to induce mesodermal gene expression. We demonstrate that a partially activated form of MAPKK restores expression of the mesodermal genes *Xcad-3* and *Xbra*, eliminated by the dominant-negative FGF receptor (ΔFGFR). Similar to the results reported earlier with ΔFGFR, expression of a dominant-negative form of MAPKK (MAPKKD) preferentially eliminates the dorsal expression of *Xcad-3* and *Xbra*. We tested whether the regional localization of bone morphogenetic protein-4 (BMP-4) could explain why both MAPKKD and ΔFGFR eliminate the dorsal and not the ventral expression of *Xcad-3* and *Xbra*. We show that ectopic expression of BMP-4 is sufficient to maintain the dorsal expression of *Xcad-3* and *Xbra* in embryos containing ΔFGFR and that expression of a dominant-negative BMP receptor reduces the dorsal–ventral differences in ΔFGFR embryos. These results indicate that regional localization of BMP-4 is responsible for the dorsal–ventral asymmetry in FGF/MAPKK-mediated mesoderm induction.

INTRODUCTION

A central focus of vertebrate developmental biologists over the past several decades has been to identify the intercellular signals that are responsible for inducing and patterning the mesoderm. In recent years, several candidate mesoderm-inducing factors have been identified, including members of the fibroblast growth factor (FGF) family and members of the transforming growth factor-β (TGF-β) family (reviewed in Kimelman *et al.*, 1992; Sive, 1993; Kessler and Melton, 1994). Other intercellular signaling molecules have been identified that pattern the mesoderm by altering the competence of the cells that respond to mesoderm-inducing agents. These include members of the *Wnt* family (reviewed in Moon *et al.*, 1993), noggin (Smith and Harland, 1992), and chordin (Sasai *et al.*, 1994).

Our goal has been to establish the signaling pathways that lead to the formation of mesoderm *in vivo*. Previous studies have indicated that the FGF receptor (Amaya *et al.*, 1991), a SH2-containing protein tyrosine phosphatase (SH-PTP2; Tang *et al.*, 1995), Ras (Whitman and Melton, 1992), and Raf (MacNicol *et al.*, 1993) are necessary for FGF-mediated mesoderm induction. Since addition of FGF to embryonic explants causes an increase in mitogen-activated protein kinase (MAPK) activation, it is likely that FGF-mediated activation of Raf leads to an elevation in MAPK activity by activating MAPK kinase (MAPKK); (Graves *et al.*, 1994; Hartley *et al.*, 1994; LaBonne and Whitman, 1994). However, Raf has been shown to have intracellular targets other than MAPKK (Li and Sedivy, 1993; Radziwill *et al.*, 1995). In addition, other intracellular proteins activated by FGF, such as phospholipase Cγ and PKC, have been shown not to be necessary for mesoderm formation (Gillespie *et al.*, 1992; Muslin *et al.*, 1994; Ryan and Gillespie, 1994). It is therefore not clear whether activation of MAPK is necessary for mesoderm induction.

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We show here that MAPKK is an important component of mesodermal induction in *Xenopus* embryos. Ectopic expression of an activated form of MAPKK expands the expression domains of two pan-mesodermal markers, *Xcad-3* and *Xbra*, and it overcomes the inhibition of dorsal *Xcad-3* and *Xbra* expression caused by a dominant-negative FGF receptor (ΔFGFR). Complementary studies by Gotoh *et al.* (1995) and LaBonne *et al.* (1995) demonstrate that an activated form of MAPKK is able to induce mesoderm in animal cap explants. In addition, we show that expression of a dominant-negative form of MAPKK selectively inhibits the expression of *Xbra* and *Xcad-3* in the dorsal region of the *Xenopus* gastrula stage embryo. These results demonstrate that the MAPK pathway is necessary and sufficient for the FGF-mediated induction of mesoderm. Furthermore, we show that the asymmetry in the response to inhibition of the FGF/MAPKK pathway can be explained by the regional localization of bone morphogenetic protein-4 (BMP-4), a member of the TGF-β family. We demonstrate that ectopic expression of BMP-4 can rescue *Xcad-3* and *Xbra* expression in the dorsal region of embryos injected with RNA encoding ΔFGFR. In addition, coexpression of ΔFGFR and a dominant-negative BMP receptor eliminates expression of *Xcad-3* and *Xbra* in both the dorsal and ventral regions of the embryo. These results demonstrate that even the relatively simple expression patterns of the pan-mesodermal markers *Xcad-3* and *Xbra* can be regulated by combinations of signaling factors and that the combination of factors responsible for expression of a gene may vary from region to region for an individual gene.

MATERIALS AND METHODS

Embryos

Fertilized eggs were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 *M* NaCl, 2 m*M* KCl, 1.0 m*M* $MgSO_4$, 2.0 m*M* $CaCl_2$, 5.0 m*M* Hepes, and 0.1 m*M* EDTA, pH 7.8). The jelly coat was removed 20 min postfertilization with 2% cysteine, pH 7.8, washed a minimum of three times in 0.1× MMR, and then incubated in 0.1× MMR at 14 to 23°C. Embryos were staged following the methods of Nieuwkoop and Faber (1967).

Construction of Transcription Vectors Containing MAPKK and MAPKK Mutants

Construction of MKK1a and of the partially-activated (S222E) and dominant-negative (S222A) mutations have been described previously (Seger *et al.*, 1992, 1994). The fragments encoding MAPKK and the corresponding mutations were excised from the original pcDNA3 vector (Invitrogen), *Bam*HI linkers were added, and the fragments were inserted into the *Bgl*II site of SP64T (Krieg and Melton, 1984).

RNA Synthesis and Microinjection

RNAs encoding MAPKK*, MAPKKD, MAPKKwt, ΔFGFR (Amaya *et al.*, 1991), ΔXBMPR (A.S. and N.U., unpublished data), and BMP-4 (Dale *et al.*, 1992) were synthesized *in vitro*. MAPKK*, MAPKKD, and MAPKKwt were linearized with *Bam*HI, ΔFGFR and ΔXBMPR were linearized with *Eco*RI, and BMP-4 was linearized with *Sal*I. RNA was synthesized using the SP6 mMessage mMachine kit (Ambion) following the manufacturer's instructions with the exception that after DNAse I treatment, the RNA was extracted with phenol:chloroform (1:1) and unincorporated nucleotides were extracted with a microcon 100 microconcentrator (Amicon). Yields were quantitated by UV spectrophotometry. RNA was diluted to the appropriate concentrations and injected as 10-nl volumes. When mixtures of RNAs were used, the components were mixed such that a single injection of 10 nl would be necessary at each injection site. Embryos at the 2- to 4-cell stage were injected on both sides of the first cleavage furrow in the marginal zone on both the dorsal and the ventral sides of the embryo (a total of four injections per embryo) following published procedures (Moon and Christian, 1989).

In Situ Hybridization and Probe Synthesis

Whole-mount *in situ* hybridization was performed using digoxigenin-labeled RNA probes (Harland, 1991), with several modifications according to Anne Knecht and Richard Harland (personal communication). In addition, the following steps were also modified: probes were not digested with sodium carbonate, the RNase digestion step was omitted, and levamisole was eliminated from the alkaline phosphate buffer. These modifications produced the same staining patterns as the original protocol; however, the stain appeared more rapidly and was more intense with little increase in background staining. The *Xcad-3* probe was synthesized from a *Eco*RV-digested *pXcad-50* construct using T3 as previously described (Northrop and Kimelman, 1994). The *Xbra* antisense probe was synthesized from an *Eco*RV digest and transcribed with T7 (Smith *et al.*, 1991).

RESULTS

Construction of MAPKK and MAPKKD Expression Vectors*

Important regulatory regions and sites of phosphorylation have been identified in several protein kinases and deletion or mutation of these sites frequently results in proteins with altered biological activity. MAPKK has previously been shown to be regulated by serine phosphorylation (Gomez and Cohen, 1991; Kyriakis *et al.*, 1992) and *in vitro* studies of MAPKK activity have suggested an essential role for serine 222 phosphorylation in activating *Xenopus* MAPKK (Gotoh *et al.*, 1994). Therefore, serine 222 of the human MAPKK (MKK1a; Seger *et al.*, 1992) was mutated to alanine (S222A) in an attempt to create a dominant-negative

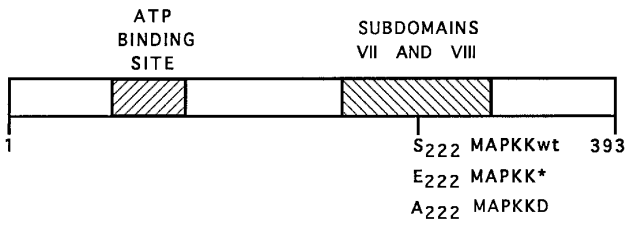


FIG. 1. Site of MAPKK mutations. Illustrated is a schematic representation of MAPKK indicating the site of the serine residue at position 222 which has been mutated to glutamic acid for MAPKK* and to alanine for MAPKKD.

MAPKK and to glutamic acid (S222E) in an attempt to constitutively activate the enzyme (Fig. 1; Seger *et al.*, 1994). These constructs were found to be dominant-negative and partially activated when expressed in NIH 3T3 cells (Seger *et al.*, 1994). MKK1a and the two mutations S222A and S222E were then inserted into the *Xenopus* RNA expression vector, SP64T (Krieg and Melton, 1984), and are referred to as MAPKKwt, MAPKKD, and MAPKK*, respectively. RNAs encoding these proteins were transcribed *in vitro* and injected into *Xenopus* embryos at the 2- to 4-cell stage in order to study the role of MAPKK in *Xenopus* mesoderm induction.

Injection of RNA Encoding MAPKK Expands the Ventral Expression of Xcad-3 and Xbra*

In order to determine whether MAPKK* is sufficient to ectopically induce expression of mesodermal genes, RNA encoding MAPKK* was injected either into the animal pole region of each blastomere of 4-cell stage *Xenopus* embryos or into the dorsal and ventral marginal zones on both sides of the first cleavage plane of 2- to 4-cell stage embryos. Expression of the pan-mesodermal markers, *Xcad-3* and *Xbra*, was then examined by *in situ* hybridization. *Xcad-3* and *Xbra* are normally expressed in a band of cells encircling the blastopore lip in gastrula stage embryos (Smith *et al.*, 1991; Northrop and Kimelman, 1994; Fig. 2A and 2A′). The expression patterns of these two genes are very similar at the early to mid-gastrula stages except that *Xcad-3* expression is somewhat fainter in the dorsal region, but this varies among batches of embryos (compare Figs. 3A and 6A). In approximately 80% of the embryos injected in either the animal pole region or in the dorsal and ventral marginal zone, the expression of both *Xcad-3* and *Xbra* expanded beyond the normal marginal zone expression pattern in the ventral, but not the dorsal, region of the embryo (Figs. 2B and 2B′). While expansion of ventral expression of *Xcad-3* and *Xbra* were observed at the same frequency, the ventral expansion of *Xcad-3* usually covered a larger area than the ventral expansion of *Xbra* expression (data not shown). Embryos that were injected with RNA encoding MAPKKwt did not exhibit any expansion of *Xcad-3* or *Xbra* expression (data not shown). Therefore, this effect required the activating mutation and was not due to the overexpression of MAPKK. These results show that MAPKK* is able to induce expression of the mesodermal markers *Xcad-3* and *Xbra* beyond their normal boundaries of expression in the ventral region of the embryo, but it cannot induce significant expression of these genes in the animal cap region or on the dorsal side of the embryo.

MAPKK Can Rescue Expression of Xcad-3 and Xbra in Embryos Co-Injected with ΔFGFR*

We examined whether MAPKK* could rescue FGF signaling during mesoderm induction in embryos expressing ΔFGFR. Embryos were injected at the 2- to 4-cell stage in the dorsal and ventral marginal zone with RNA encoding MAPKK*, ΔFGFR, or a mixture of MAPKK* and ΔFGFR. Embryos treated with ΔFGFR exhibited reduced expression of *Xcad-3* and *Xbra* in the dorsal marginal region of the embryos as previously described (Northrop and Kimelman, 1994; Figs. 3C and 3C′ and Table 1). When MAPKK* was co-injected with ΔFGFR, dorsal expression of both *Xcad-3* and *Xbra* was rescued (Figs. 3D and 3D′ and Table 1). MAPKK* was more efficient at rescuing the ΔFGFR-mediated inhibition of *Xbra* than is apparent in Table 1. Although co-injection of MAPKK* did not always restore complete *Xbra* expression, embryos injected with MAPKK* and ΔFGFR consistently had a smaller region lacking *Xbra* expression than was observed in embryos injected with ΔFGFR alone, demonstrating that some degree of rescue occurred.

Embryos were also injected with RNA encoding MAPKKwt or a mixture of MAPKKwt and ΔFGFR to determine whether the effects we observed were specifically due to the expression of an activated form of MAPKK or were due to an increase in the levels of MAPKK expression. Injection of RNA encoding MAPKKwt was unable to rescue expression of *Xcad-3* and *Xbra* in embryos co-injected with ΔFGFR (Table 1). Furthermore, we did not observe any partial rescue of *Xbra* expression in ΔFGFR embryos co-injected with MAPKKwt as discussed above for embryos co-injected with MAPKK*. These experiments demonstrate that the introduction of an activated form of MAPKK is able to specifically overcome the inhibition of expression of *Xcad-3* and *Xbra* caused by ΔFGFR.

Injection of RNA Encoding MAPKKD Disrupts the Dorsal Expression of Xcad-3 and Xbra

Since an activated MAPKK can rescue a block in the FGF signal transduction pathway, we next examined whether a dominant-negative construct of MAPKK could cause the same effects on mesodermal gene expression as the dominant-negative FGF receptor. RNA encoding MAPKKD was injected into the dorsal and ventral marginal zone of embryos at the 2- to 4-cell stage on both sides of the first cleavage plane. MAPKKD inhibited expression of *Xcad-3* and *Xbra* preferentially in the dorsal region of the embryo (Figs. 4C and 4C′). In a total of 18 independent experiments,

Xcad-3 expression was eliminated from the dorsal region of gastrula stage embryos in 49% of the samples (157/320) while ventral reduction of expression was never observed (data not shown). In embryos stained for *Xbra* expression, dorsal staining was eliminated in 63% of the embryos (198/313) while ventral reduction of expression was seen in only 3.5% of the embryos (11/313; data not shown). These results are similar to the effect seen in embryos expressing ΔFGFR (Northrop and Kimelman, 1994).

To demonstrate that the elimination of gene expression was a specific effect of MAPKKD, RNAs encoding MAPKK* and MAPKKD were co-injected in the dorsal and ventral marginal zone of 2- to 4-cell stage embryos. MAPKK* was able to effectively rescue the dorsal reduction of *Xcad-3* and *Xbra* expression in embryos expressing MAPKKD (Figs. 4D and 4D′ and Table 2). As a further control, RNA encoding MAPKKwt was injected both alone and in combination with MAPKKD. MAPKKwt did not reduce expression of *Xcad-3* and *Xbra* when injected alone, and when co-injected with MAPKKD, it was unable to rescue mesodermal gene expression (data not shown). These results demonstrate that the elimination of dorsal expression of *Xcad-3* and *Xbra* is due to a specific effect of MAPKKD and that MAPKKD can produce the same types of changes in gene expression as was observed with ΔFGFR.

BMP-4 Rescues the Dorsal Expression of Xcad-3 and Xbra in ΔFGFR-Injected Embryos

Our previous study (Northrop and Kimelman, 1994), and the work discussed above, demonstrated that the *Xenopus* embryo has a dorsal–ventral asymmetry in its response to inhibition of FGF signaling through the MAPKK pathway. One possible explanation for the dorsal–ventral difference is the regional localization of another factor which affects the response of *Xcad-3* and *Xbra* to inhibition of the FGF/MAPKK pathway. For example, a ventralizing factor such as BMP-4 (Koster *et al.,* 1991; Dale *et al.,* 1992; Jones *et al.,* 1992) could act synergistically with FGF, resulting in an increased resistance of *Xcad-3* and *Xbra* ventral expression to the effects of ΔFGFR. It has recently been determined that BMP-4 transcripts are localized to the ventral and lateral regions of the gastrula stage embryo, but are absent from the dorsal side (Fainsod *et al.,* 1994; Schmidt *et al.,* 1995), making BMP-4 an excellent candidate for a regionally localized molecule affecting FGF/MAPKK signaling.

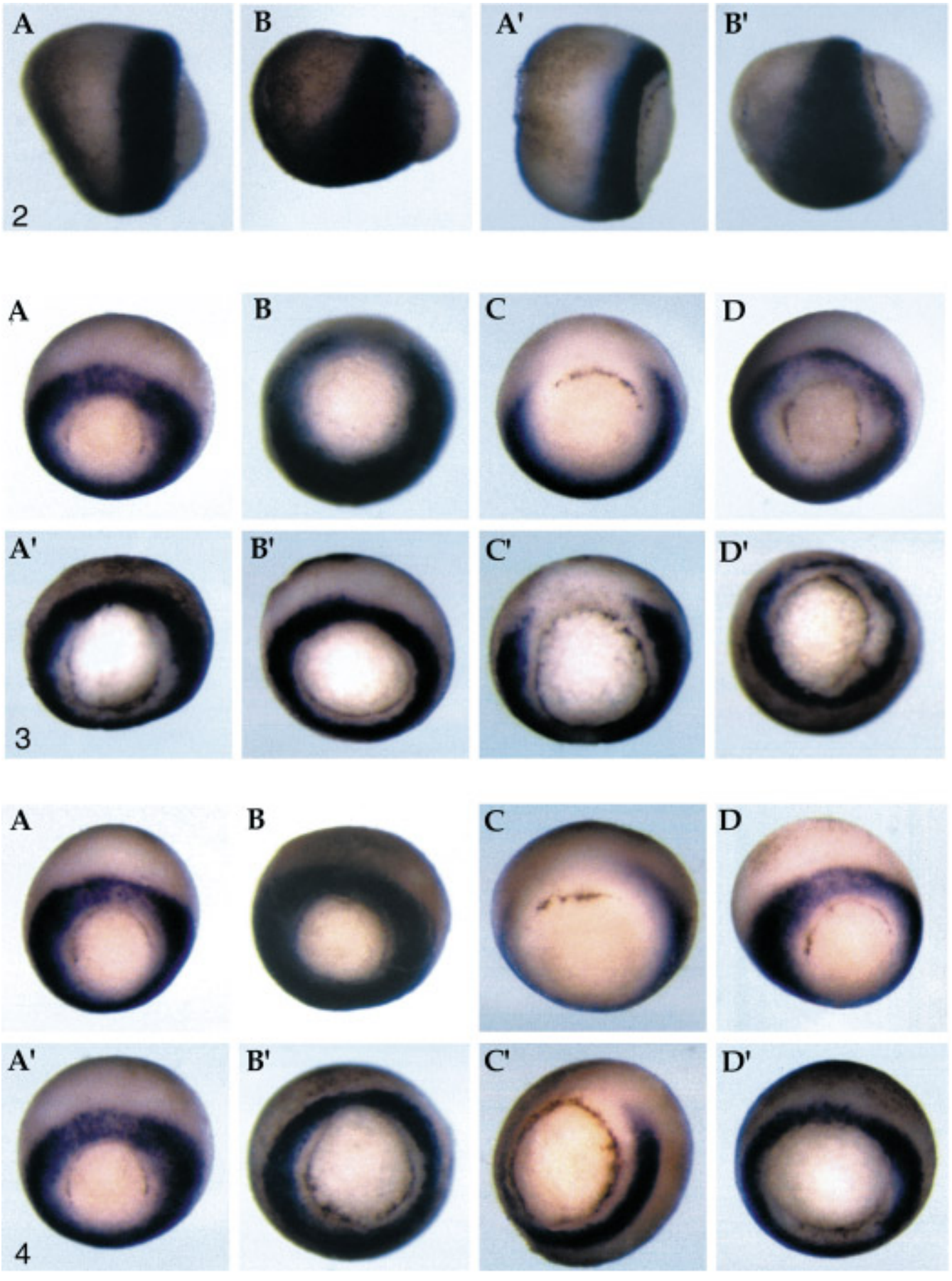
In order to determine whether BMP-4 is responsible for the dorsal–ventral differences observed for FGF- and MAPKK-mediated signaling, we decided to use RNA encoding ΔFGFR rather than MAPKKD. We have demonstrated above that MAPKK is part of the mesoderm-inducing signal transduction cascade activated by FGF and that blocking the pathway at either step produces the same results on *Xcad-3* and *Xbra* expression. Since MAPKKD required a 5- to 10-fold higher dose of RNA than ΔFGFR to eliminate *Xcad-3* and *Xbra* expression from the dorsal region of the embryo, it was easier to inject combinations of RNA using ΔFGFR.

To characterize the effects of BMP-4 in the presence of ΔFGFR, RNA encoding BMP-4, ΔFGFR, or a mixture of the two was injected into the dorsal and ventral marginal zone of a 2- to 4-cell stage embryo. The embryos were then scored during the mid-gastrula stages for expression of *Xcad-3* and *Xbra*. Injection of BMP-4 alone expanded the marginal zone expression of both genes in both the dorsal and the ventral regions of the embryo and occasionally expanded the expression throughout the entire animal hemisphere (Schmidt *et al.,* 1995; data not shown). Injection of ΔFGFR eliminated dorsal expression of *Xcad-3* and *Xbra* in 96% of each of the samples (Table 3). Co-injection of BMP-4 with ΔFGFR RNAs effectively rescued expression of both genes but was somewhat more efficient at rescuing *Xcad-3* than *Xbra* expression (Figs. 5D and 5D′ and Table 3). BMP-4 rescued over 80% of the dorsal *Xcad-3* expression in embryos injected with ΔFGFR, whereas only about 50% of the embryos stained for *Xbra* expression were rescued by BMP-4 co-injection (Table 3). In addition, even when *Xbra* expression was rescued in the dorsal region of the embryo, the rescue was

FIG. 2. Injection of RNA encoding MAPKK* expands the ventral expression patterns of *Xcad-3* and *Xbra* in gastrula stage embryos. Embryos at the 2- to 4-cell stage were injected in both the dorsal and ventral marginal zones with RNA encoding MAPKK* and stained for *Xcad-3* (A and B) and *Xbra* (A′ and B′) expression at the gastrula stage by *in situ* hybridization. (A and A′) Uninjected embryos (B and B′), embryos injected with 5 ng RNA encoding MAPKK*. The embryos are oriented with dorsal toward the top of the page and anterior toward the left.

FIG. 3. RNA encoding MAPKK* injected in both the dorsal and ventral marginal zones can rescue expression of *Xcad-3* and *Xbra* in embryos co-injected with ΔFGFR. Uninjected embryos (A and A′), embryos injected with 5 ng RNA encoding MAPKK* (B and B′), embryos injected with 1 ng RNA encoding ΔFGFR (C and C′), and embryos co-injected with 5 ng RNA encoding MAPKK* and 1 ng RNA encoding ΔFGFR (D and D′) were fixed at the gastrula stage and stained for *Xcad-3* (A–D) and *Xbra* (A′–D′) expression by *in situ* hybridization. Vegetal view, with the dorsal side oriented toward the top of the page. Note that the ventral expansion in embryos injected with MAPKK* is difficult to see in a vegetal view and is frequently less pronounced in embryos stained for *Xbra* than for *Xcad-3* (see text).

FIG. 4. Injection in both the dorsal and ventral marginal zones with RNA encoding MAPKKD disrupts expression of *Xcad-3* and *Xbra* in a dorsal–ventral-dependent manner, an effect which is blocked by coexpression of MAPKK*. Uninjected embryos (A and A′), embryos injected with 2 ng RNA encoding MAPKK* (B and B′), embryos injected with 5 ng RNA encoding MAPKKD (C and C′), and embryos co-injected with 2 ng RNA encoding MAPKK* and 5 ng RNA encoding MAPKKD (D, D′) were fixed at the gastrula stage and stained for *Xcad-3* (A–D) and *Xbra* (A′–D′) expression by *in situ* hybridization. Vegetal view, with the dorsal side oriented toward the top of the page.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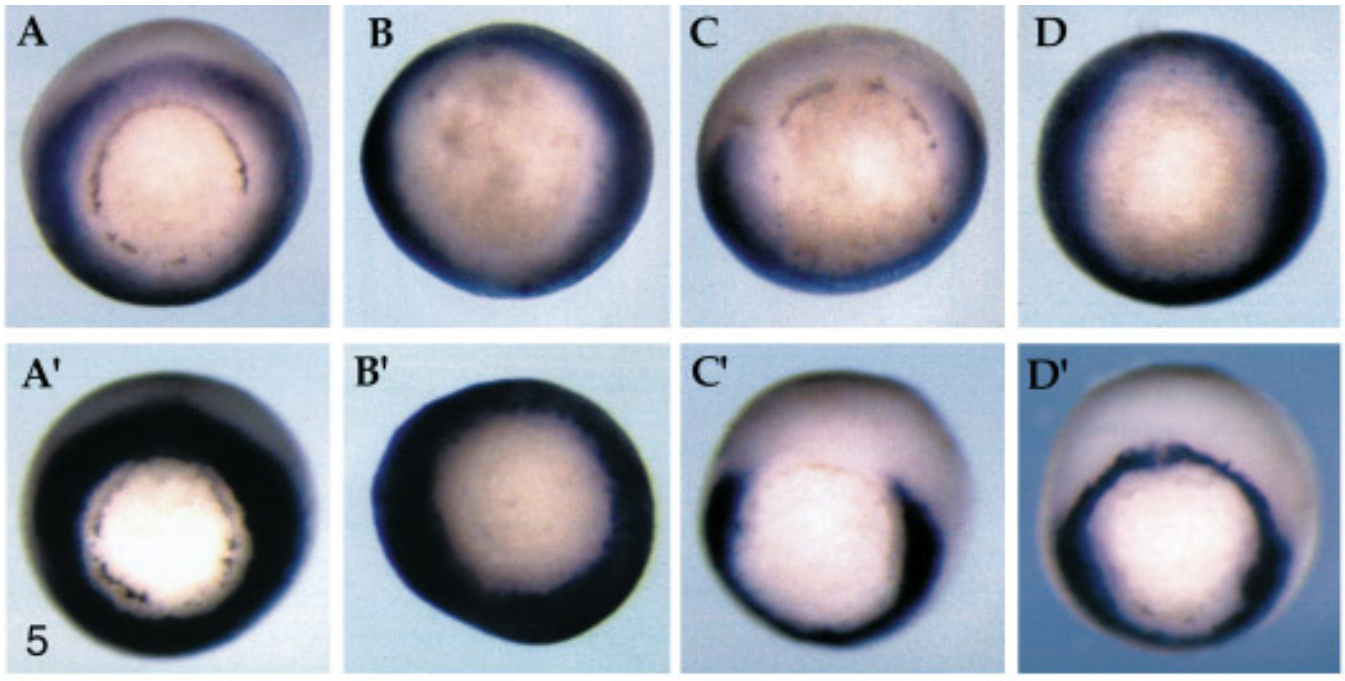

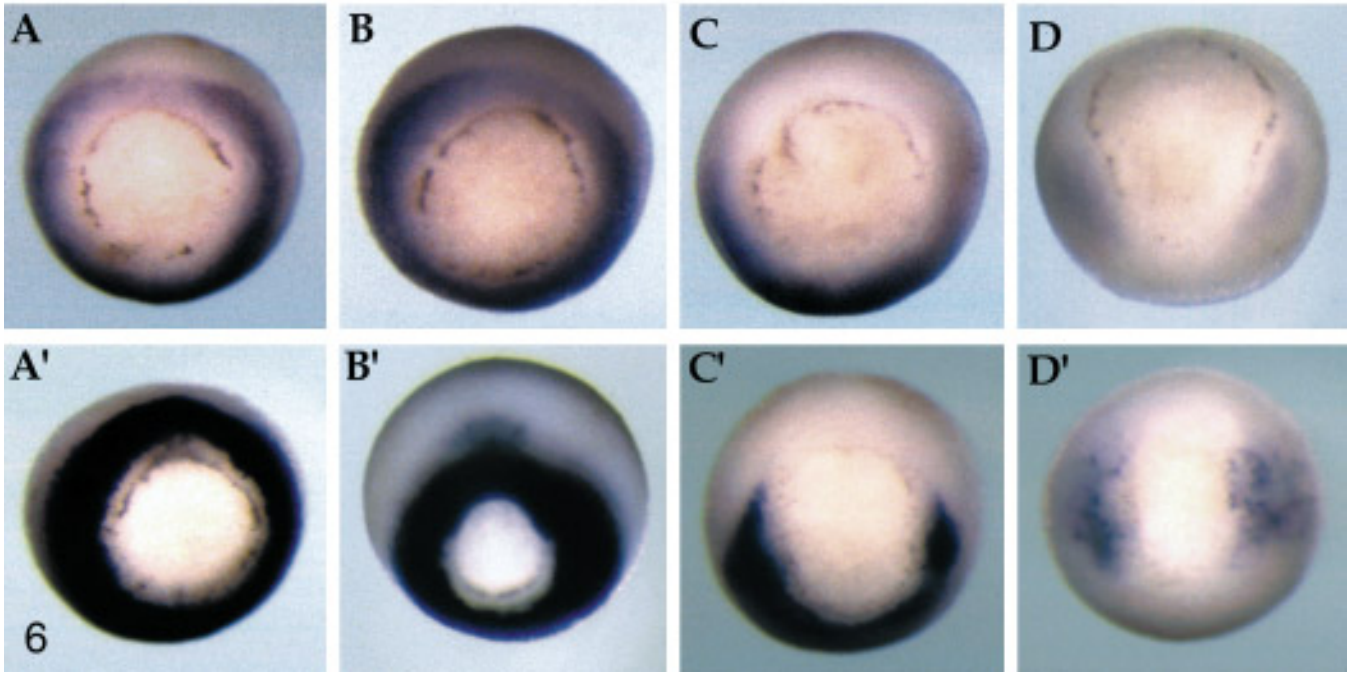

FIG. 5. BMP-4 rescues the dorsal expression of *Xcad-3* and *Xbra* eliminated in ΔFGFR-injected embryos when both are co-injected into the dorsal and ventral marginal zones. Uninjected embryos (A and A′), embryos injected with 1 ng RNA encoding BMP-4 (B and B′), embryos injected with 1 ng RNA encoding ΔFGFR (C and C′), and embryos co-injected with 1 ng RNA encoding BMP-4 and 1 ng RNA encoding ΔFGFR (D and D′) were fixed at the gastrula stage and stained for *Xcad-3* (A–D) and *Xbra* (A′–D′) expression by *in situ* hybridization. Vegetal view, with the dorsal side oriented toward the top of the page. Note that BMP-4 caused an expansion of *Xcad-3* and *Xbra* expression in the dorsal and ventral animal hemispheres in these embryos which are not observed in this orientation (B and B′).

FIG. 6. RNA encoding ΔXBMPR injected in both the dorsal and ventral marginal zones eliminates the dorsal–ventral gradient of sensitivity to ΔFGFR. Uninjected embryos (A and A′), embryos injected with 1 ng RNA encoding ΔXBMPR (B and B′), embryos injected with 1 ng RNA encoding ΔFGFR (C and C′), and embryos co-injected with 1 ng RNA encoding ΔXBMPR and 1 ng RNA encoding ΔFGFR (D and D′) were fixed at the gastrula stage and stained for *Xcad-3* (A–D) and *Xbra* (A′–D′) expression by *in situ* hybridization. Vegetal view, with the dorsal side oriented toward the top of the page.

TABLE 1
MAPKK* Rescues *Xcad-3* and *Xbra* Expression in Embryos Expressing the Truncated FGF Receptor

	Xcad-3[a]			*Xbra*[b]		
RNA-injected	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain
None	22	0	0%	159	0	0%
5 ng MAPKK*	57	0	0%	167	0	0%
1 ng ΔFGFR	9	33	79%	15	142	90%
1 ng ΔFGFR + 5 ng MAPKK*	33	3	8%	115	72	39%
MAPKKwt	22	0	0%	36	0	0%
1 ng ΔFGFR + 5ng MAPKKwt	1	25	96%	6	51	90%

Note. Embryos were injected at the two-cell stage in the dorsal and ventral marginal zone with RNA and then harvested at stage 12 (midgastrula) and stained for *Xcad-3* or *Xbra* expression by *in situ* hybridization.
[a] Data compiled from two experiments (one experiment included MAPKKwt as a control).
[b] Data compiled from seven experiments (two experiments included MAPKKwt as a control).

less efficient than the rescue of dorsal *Xcad-3* expression (Figs. 5D and 5D′). These results demonstrate that ectopic expression of BMP-4 can rescue the dorsal elimination of *Xcad-3* and *Xbra* expression caused by the dominant-negative FGF receptor and suggest that the ventral region of the embryo normally requires a lower level of FGF signaling to maintain *Xcad-3* and *Xbra* expression due to the localization of BMP-4 transcripts to that region.

RNA Encoding a Dominant-Negative BMP Receptor Eliminates the Dorsal–Ventral Gradient of Sensitivity to ΔFGFR

While ectopic expression of BMP-4 can rescue the dorsal–ventral asymmetry when FGF signaling is blocked, these results do not demonstrate whether this is a role of endogenous BMP-4. To examine whether disruption of the BMP-4 signal transduction pathway in whole embryos could eliminate the dorsal–ventral differences in FGF-mediated mesoderm induction, a *Xenopus* dominant-negative BMP receptor (ΔXBMPR; A.S. and N.U., unpublished results) was co-injected with ΔFGFR. This truncated BMP receptor blocks BMP-4 signaling but has little effect on other TGF-β family members such as activin (Schmidt *et al.*, 1995; see also Graff *et al.*, 1994). If BMP-4 signaling is responsible for maintaining *Xbra* and *Xcad-3* expression in the ventral region of ΔFGFR-injected embryos, we would expect that disruption of the BMP-4 signaling pathway would eliminate the dorsal–ventral asymmetry. Therefore in embryos injected with both ΔFGFR and ΔXBMPR, *Xcad-3* and *Xbra*

TABLE 2
MAPKKD Disrupts Dorsal Expression of *Xcad-3* and *Xbra*

	Xcad-3[a]			*Xbra*[a]		
RNA-injected	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain
No RNA	26	0	0%	42	0	0%
2 ng MAPKK*	50	0	0%	50	0	0%
5 ng MAPKKD	22	17	44%	11	13	54%
2 ng MAPKK* + 5 ng MAPKKD	27	2	7%	30	2	6%

Note. Embryos were injected at the two-cell stage in the dorsal and ventral marginal zone with RNA and then harvested at stage 12 (midgastrula) and stained for *Xcad-3* or *Xbra* expression by *in situ* hybridization.
[a] Data compiled from two experiments.

TABLE 3

BMP4 Rescues Dorsal Expression of *Xcad-3* and *Xbra* in Embryos Expressing ΔFGFR

	Xcad-3[a]			*Xbra*[b]		
RNA-injected	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain	Complete ring of stain	Missing dorsal stain	% Missing dorsal stain
No RNA	118	0	0%	126	0	0%
1 ng BMP4	88	0	0%	81	0	0%
1 ng ΔFGFR	4	85	96%	3	76	96%
1 ng BMP4 + 1 ng ΔFGFR	75	10	12%	50	44	47%

Note. Embryos were injected at the two-cell stage in the dorsal and ventral marginal zone with RNA and then harvested at stage 12 (midgastrula) and stained for *Xcad-3* or *Xbra* expression by *in situ* hybridization.

[a] Data compiled from five experiments.

[b] Data compiled from seven experiments.

expression should be inhibited equally on the dorsal and ventral sides of the embryo.

RNA encoding either ΔXBMPR, ΔFGFR, or a mixture of the two was injected into the dorsal and ventral marginal zones of *Xenopus* embryos at the 2-cell stage. Injection of ΔXBMPR alone rarely altered either *Xcad-3* or *Xbra* expression during gastrulation (Suzuki *et al.*, 1994; Schmidt *et al.*, 1995; Figs. 6B and 6B′). In 6 out of 131 embryos examined, there was some minor reduction in dorsal *Xcad-3* stain, and in 1 embryo out of 108 examined, there was also a disruption in *Xbra* staining (Table 4). It is difficult to determine whether there was any significance to this effect since it was so rarely observed, yet it was not seen in any of the control embryos. Injection of ΔFGFR eliminated *Xcad-3* and *Xbra* expression preferentially from the dorsal region of the embryo (Figs. 6C and 6C′) and also reduced ventral *Xbra* expression in a small subset of the embryos (Table 4). Injection of ΔXBMPR and ΔFGFR together resulted in elimination of mesodermal gene expression in both the dorsal and the ventral regions of the embryo (Figs. 6D and 6D′). Since the RNA injections are specifically targeted to the dorsal and ventral sides, there is often some faint lateral expression of *Xcad-3* and *Xbra* remaining in these embryos (Figs. 6D and 6D′), but expression is frequently completely eliminated. None of the embryos injected with ΔFGFR RNA alone had reduced ventral *Xcad-3* expression, while 66% of the embryos injected with both ΔXBMPR and ΔFGFR had *Xcad-3* expression eliminated from both the

TABLE 4

ΔXBMPR Eliminates the Dorsal–Ventral Differences in *Xcad-3* and *Xbra* Expression in Embryos Expressing ΔFGFR

	Xcad-3[a]					*Xbra*[b]				
RNA-injected	Complete ring of stain	Missing dorsal stain	Missing dorsal and ventral stain	% Missing dorsal stain	% Missing dorsal and ventral stain	Complete ring of stain	Missing dorsal stain	Missing dorsal and ventral stain	% Missing dorsal stain	% Missing dorsal and ventral stain
no RNA	108	0	0	0%	0%	122	0	0	0%	0%
1 ng ΔXBMPR	125	6	0	5%	0%	107	1	0	1%	0
1 ng ΔFGFR	33	94	0	74%	0%	3	89	13	85%	12%
1 ng ΔXBMPR + 1 ng ΔFGFR	1	66	131	33%	66%	4	30	94	23%	73%

Note. Embryos were injected at the two-cell stage in the dorsal and ventral marginal zone with RNA and then harvested at stage 12 (midgastrula) and stained for *Xcad-3* or *Xbra* expression by *in situ* hybridization.

[a] Data compiled from five experiments.

[b] Data compiled from seven experiments.

dorsal and the ventral regions (Table 4). In embryos stained for *Xbra* expression, 73% exhibited both a dorsal and ventral reduction in stain when co-injected with ΔXBMPR and ΔFGFR, whereas only 12% lacked *Xbra* expression in both regions when injected with RNA encoding ΔFGFR alone (Table 4). These results demonstrate that coexpression of a dominant-negative BMP receptor reduces the dorsal–ventral asymmetry of FGF-mediated mesoderm induction.

DISCUSSION

MAPKK Is Responsible for FGF-Mediated Induction of Xcad-3 and Xbra Expression

In this report we have shown that MAPKK is necessary and sufficient for FGF-mediated mesoderm induction of *Xcad-3* and *Xbra* in the *Xenopus* embryo. We have demonstrated that ectopic expression of MAPKK* is sufficient to rescue the elimination of *Xcad-3* and *Xbra* expression caused by ΔFGFR. This effect is due to the activating mutation (serine to glutamic acid), since overexpression of MAPKKwt did not rescue mesodermal gene expression in embryos expressing ΔFGFR. In complementary studies, activated forms of MAPKK (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995) and MAPK (LaBonne *et al.*, 1995) were shown to induce expression of *Xbra* in animal caps.

We have demonstrated that MAPKK is necessary for mesoderm induction by ectopically expressing RNA encoding MAPKKD and eliminating the dorsal expression of the mesodermal genes *Xcad-3* and *Xbra*. The preferential elimination of dorsal expression of these two mesodermal markers is the same effect observed when RNA encoding ΔFGFR is expressed. This elimination of *Xcad-3* and *Xbra* expression is a specific effect of the MAPKKD mutation (serine to alanine) since expression of neither MAPKKwt or MAPKK* reduced the expression of *Xcad-3* and *Xbra*. In addition, expression of these two mesodermal markers are rescued by coexpressingg RNA encoding MAPKK* with MAPKKD RNA, indicating that this effect is due to interference with MAPKK signaling and not due to nonspecific effects of RNA overexpression. Further evidence that MAPKK and MAPK are necessary for mesoderm induction comes from the studies of Gotoh *et al.* (1995) and LaBonne *et al.* (1995). Both groups demonstrated that a MAPK-specific phosphatase can block the FGF-mediated formation of mesoderm in animal cap explants and produce embryos with a similar phenotype to that caused by ectopic expression of ΔFGFR.

Regional Localization of BMP-4 Contributes to the Dorsal–Ventral Asymmetry of FGF-Mediated Mesoderm Induction

The dorsal–ventral differences in the response of *Xcad-3* and *Xbra* expression to perturbations in the FGF/MAPKK pathway are not likely to be due to asymmetric distribution of FGF or its receptor since there is no apparent difference in the concentration of bFGF or the endogenous FGF receptor (*flg*) between the dorsal and ventral regions of the *Xenopus* embryo (Ding *et al.*, 1992; Song and Slack, 1994). In addition, as shown in this report, MAPKK exhibits a similar dorsal–ventral asymmetry in its ability to effect *Xcad-3* and *Xbra* expression. Finally, MAPK is activated to a similar extent by FGF in animal cap explants isolated from either the dorsal or the ventral regions of the embryo (L. Graves, J.N., E.K., and D.K., unpublished results). These results indicate that the dorsal–ventral differences observed in FGF-mediated mesoderm induction are not likely to be due to asymmetrically distributed components of the FGF signal transduction pathway, but rather that the observed dorsal–ventral differences might be due to the presence of another asymmetrically active factor.

One possibility is that a ventrally localized factor could enhance *Xcad-3* and *Xbra* expression and thereby cause expression of these two genes to be more resistant to disruptions in the FGF/MAPKK signaling pathway in the ventral region of the embryo. Recent studies have shown that BMP-4 transcripts are localized to the lateral and ventral regions of the gastrula stage embryo but are absent from the dorsal region (Fainsod *et al.*, 1994; Schmidt *et al.*, 1995). Therefore, BMP-4 is a strong candidate to regionally affect the ability of FGF to induce and maintain *Xcad-3* and *Xbra* expression.

Our results indicate that endogenous BMP-4 reduces the level of FGF signaling that is necessary for *Xcad-3* and *Xbra* expression. When FGF signaling is disrupted by expression of ΔFGFR, the dorsal mesoderm, which lacks BMP-4 transcripts, is no longer able to maintain *Xcad-3* and *Xbra* expression. This inhibitory effect of ΔFGFR can be overcome by ectopic expression of BMP-4. Furthermore, while expression of a dominant-negative BMP receptor alone rarely affected *Xcad-3* and *Xbra* expression patterns, co-injection with ΔFGFR eliminated *Xcad-3* and *Xbra* transcripts from both the dorsal and ventral regions of the gastrula stage *Xenopus* embryo. These results indicate that the dorsal–ventral differences in the sensitivity of *Xcad-3* and *Xbra* to ΔFGFR can be explained by the absence of BMP-4 from the dorsal region of the embryo.

Differential Regulation of Xbra and Xcad-3 by FGF

Although both *Xcad-3* and *Xbra* are preferentially eliminated from the dorsal side of the embryo by ΔFGFR, *Xbra* expression is disrupted at lower doses of ΔFGFR RNA than is *Xcad-3* expression (Northrop and Kimelman, 1994). These results suggest that the expression of *Xbra* may require a higher level of FGF signaling. In accordance with this hypothesis, we find that both MAPKK* and BMP-4 rescue the elimination of *Xcad-3* expression by ΔFGFR more efficiently than they rescue *Xbra* expression (see Tables 1 and 3 and compare the dorsal region of *Xcad-3* and *Xbra* expression in Figs. 3D and D′ and 5D and 5D′). In addition, MAPKK* is able to expand the ventral region of *Xcad-3* expression to a greater extent than it expands ventral *Xbra* expression. This observed difference in the requirements for FGF signaling levels for maintaining *Xcad-3* and *Xbra* expression may be due to an additional factor(s)

that helps maintain *Xcad-3* expression at lower levels of FGF signaling. In support of this hypothesis, we note that *Xbra* is an immediate-early response gene when induced by FGF (Smith *et al.*, 1991), whereas *Xcad-3* requires additional protein synthesis (J.N. and D.K., unpublished data). It is unlikely that *Xbra* is the additional factor that is required for *Xcad-3* expression since the expression of *Xbra* can be eliminated by doses of ΔFGFR that continue to maintain *Xcad-3* expression. The additional factor is also unlikely to be BMP-4 since differences between the levels of FGF signaling needed to maintain *Xcad-3* and *Xbra* expression are observed in the dorsal region of the embryo, which does not contain BMP-4 transcripts. Therefore it is likely that there is yet another factor, acting in combination with FGF, that helps maintain *Xcad-3*, but not *Xbra*, expression.

Combinatorial Signaling

This study emphasizes two important concepts. First, while addition of a single signaling factor may induce expression of a gene in an animal cap explant, the normal expression of a gene may depend on a combination of factors, and that combination may vary in different regions of the embryo. For example, while *Xcad-3* and *Xbra* expression is regulated by FGF in both the dorsal and the ventral regions of the embryo, BMP-4 also is involved in the ventral regulation of these genes. Second, our results emphasize the importance of examining the effects of multiple signaling pathways on gene expression. If this study had been limited to examining the effect of ΔXBMPR on *Xcad-3* and *Xbra* expression, we might have concluded that endogenous BMP-4 signaling does not regulate expression of these two genes. Only by blocking both signaling pathways did it become clear that both BMP-4 and FGF are important for *Xcad-3* and *Xbra* mesodermal expression and that the localization of BMP-4 imposes a differential response to FGF-mediated mesoderm induction. This level of complexity in induction and patterning of the mesoderm can only be revealed by examining the effects of multiple signaling pathways in the context of the whole embryo.

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